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Recombinant Supercharged Polypeptides Restore and Improve Biolubrication

Deepak H. Veeregowda, Anke Kolbe, Henny C. van der Mei, Henk J. Busscher, Andreas Herrmann, and Prashant K. Sharma*

Biomacromolecules do not only fulfil complex functions inside the cell or within membranes, but proteinaceous materials may also play a very critical role at interfaces. One such example is biolubrication, where sliding surfaces coated with synthetic polymers and biological building blocks have been intensively investigated.^[1] Biolubrication is an essential feature of health and can become impaired in the elderly or diseased.^[2] Sjögren's syndrome, for instance, is a disease causing a variety of symptoms like dry eyes,^[3] dry mouth,^[4] vaginal dryness^[5] and excessive friction and wear at the knee and hip joints.^[6] Biolubrication is mediated by glandular secretions containing (glyco-) proteins that adsorb at the sliding interface and form a conditioning film. Although water forms the basis of all biolubrication phenomena, it is easily removed from in between sliding surfaces during physiological activities associated with high contact pressures. To counter this, conditioning films providing biolubrication contain different glycoproteins that retain water molecules to generate repulsive hydration forces at the interface of the sliding surfaces.^[7,8] Oral lubrication by adsorbed salivary conditioning films (SCFs)^[9] is essential to facilitate speaking and mastication and protects against wear due to erosion^[10] and abrasion.^[11] Maintenance of adequate biolubrication in the oral cavity is not only challenged by disease and aging, but also by high contact pressures. Contact pressures on molar surfaces during mastication can be as high as 86 MPa^[12] which is one order of magnitude higher than the pressures experienced in hip and knee joints.^[13] This load makes the maintenance and restoration of lubrication more challenging in the oral cavity than in other parts of the human body where articulating surfaces are involved.

Often, oral dryness is due to insufficient retention of water molecules in adsorbed SCFs due to low salivary flow rates ($< 1 \text{ mL min}^{-1}$) or dysfunction of a particular salivary gland.^[2]

Patients suffering from oral dryness symptoms are treated with artificial salivas, often containing lubricants like pig gastric mucins, polyacrylic acid and carboxymethyl cellulose.^[14,15] However, artificial salivas only yield temporary relief in patients, as the adsorbed conditioning films are unable to sufficiently retain water due to lack of structural integrity.

Intrigued by the facts that cationic polyelectrolytes are able to improve the mechanical strength of polysaccharide multilayers^[16] and can form polymer-brush like structures,^[17] we tested their ability to act as an additive to improve oral lubrication. Among the existing polyelectrolytes, recombinant supercharged, unfolded polypeptides (SUPs) containing the elastin motif constitute an attractive group of proteinaceous materials that may assist in restoring biolubrication. Not only can cationic SUPs interact with the negatively charged, naturally occurring mucins, they also possess significantly lower cytotoxicity than other cationic polyelectrolytes commonly used in biomedical applications.^[18] Moreover, they are well-defined with respect to their length, composition and charge density and are broken down into non-toxic, naturally occurring amino-acids upon digestion. However, the potential of SUPs to improve biolubrication and mechanical strength of naturally occurring conditioning films has never been investigated.

In this study, we evaluated the role of SUPs with 36 (K36) and 72 (K72) positive charges in modifying the lubrication of adsorbed SCFs. A quartz crystal microbalance with dissipation monitoring (QCM-D) was used to examine changes in SCF structure after K36 and K72 adsorption and renewed exposure to saliva i.e. secondary SCF or S-SCF. Colloidal probe atomic force microscopy (AFM) was applied to determine friction and repulsive force characteristics and topography of the S-SCFs. Finally, X-ray photoelectron spectroscopy (XPS) was used to determine their degree of glycosylation. Cationic recombinant SUPs as additives recruited lubricious proteins, increased the degree of glycosylation and strength in the S-SCFs to restore and improve the lubrication under conditions of reduced availability of naturally occurring proteins.

Cationic SUPs based on elastin-like polypeptides consist of repeats of five amino acids, containing the aliphatic residues glycine (G), valine (V), proline (P), and positively charged lysine (K). Two variants with the amino acid sequences GAGP[(GVGVP)(GKGVP)₉]₄GWPH₆ (K36) and GAGP[(GVGVP)(GKGVP)₉]₈GWPH₆ (K72) were fabricated by recombinant protein expression in *Escherichia coli*. The gene sequence and respective amino acid sequence of the monomer are shown in Figure S1 of Supporting Information. The gene length was characterized using gel electrophoresis (Supporting Information, Figure S2). Typical yields were 45 mg (K36) and

Prof. A. Herrmann, A. Kolbe
Department of Polymer Chemistry
Zernike Institute for Advanced Materials
University of Groningen
Nijenborgh 4, 9747 AG Groningen, The Netherlands
D. H. Veeregowda, Prof. H. C. van der Mei,
Prof. H. J. Busscher, Dr. P. K. Sharma
University of Groningen and University Medical Center Groningen
Department of Biomedical Engineering
W.J. Kolff Institute
Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
E-mail: p.k.sharma@umcg.nl



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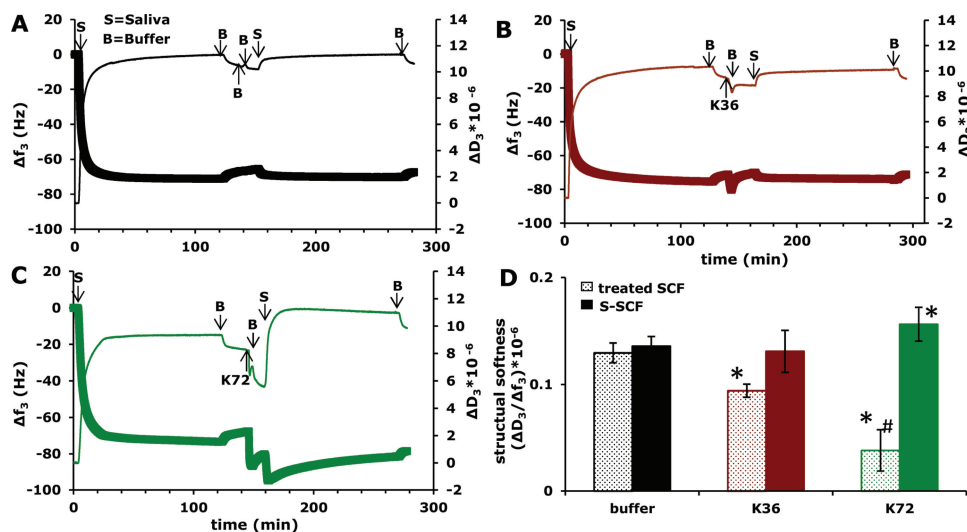


Figure 1. Influence of adsorption of recombinant cationic SUPs and renewed exposure to saliva on the softness of salivary conditioning films. (a, b, c) Examples of the QCM-D response as a function of time to protein adsorption from saliva on Au-coated quartz crystal surfaces, subsequent adsorption of cationic recombinant SUPs (2 min) and renewed exposure to salivary proteins, expressed as changes in third harmonic frequency (Δf_3 , thick line) and dissipation (ΔD_3 , thin line), together with structural softness of the adsorbed films: a) buffer/no recombinant SUP adsorption; b) adsorption of recombinant K36 and c) adsorption of recombinant K72. (d) Structural softness of salivary conditioning films after buffer treatment or recombinant cationic SUP adsorption i. e. treated SCF and after renewed exposure to saliva i.e. secondary SCF (S-SCF). Error bars represent the standard deviation over five independent measurements. Statistically significant ($p < 0.05$, two tailed Student t-test) differences in softness of films with adsorbed K36 or K72 with respect to control films are indicated by *. Significant differences in softness between films with adsorbed K72 and K36 are indicated by #.

40 mg (K72) of purified protein per liter of culture. Purified polypeptides were separated on a SDS-PAGE gel (Supporting Information, Figure S3a). Mass spectra yielded sharp peaks for both variants i.e. K36 and K72 (Supporting Information, Figure S3b and S3c) and their masses were $18,932 \pm 20$ Da for K36 and $36,330 \pm 30$ Da for K72.

The formation of SCFs on gold (Au) coated quartz crystals and the effects of their exposure to recombinant K36 and K72 or buffer, followed by renewed adsorption of salivary proteins, were observed real-time in the QCM-D, as presented in Figure 1a-c. Exposure of an existing SCF to buffer (Figure 1a) yielded a small change in the oscillating sensor frequency (Δf_3) and dissipation (ΔD_3), whereas exposure to K36 (Figure 1b) and K72 (Figure 1c) solutions caused significant decreases in Δf_3 and ΔD_3 that were largest for K72. Subsequent removal of the protein solution by perfusing the QCM-D chamber with buffer indicated a structural decrease in the softness of the SCFs, expressed as the ratio ($\Delta D_3/\Delta f_3$). Again, this effect was larger after exposure to K72 solution than after exposure to K36 solution (Figure 1d). Renewed salivary exposure over the SCFs was initiated immediately after treatment with buffer or recombinant protein solutions to form S-SCFs (Figure 1a-c) because such experimental conditions reflect best the in vivo situation of immediate reflow of saliva in the oral cavity. Renewed perfusion of the QCM-D chamber with saliva did not affect the structural softness of the S-SCF with only buffer without recombinant SUPs (Figure 1d), but S-SCF with K36 solution became softer again to a level comparable to S-SCF without recombinant SUPs. The S-SCFs with K72, however, were significantly ($p < 0.05$, two tailed Student t-test) softer than S-SCFs with K36. Bare Au-coated QCM crystals have a smooth surface

(Figure S4a), presenting uneven, globular structures upon adsorption of salivary proteins (Figure S4b), with maximal heights of around 22 nm. Similar structures are observed on S-SCFs with K36 or K72, but their heights differ considerably, from 16 nm for films comprising K36 to 32 nm when K72 is involved (Figures S4c,d).

In a next step, the lubrication properties of the SUP-modified films were investigated by colloidal probe AFM. Friction forces on bare Au-coated crystals increased linearly ($R^2 = 0.95$) with normal force up to 35 nN, corresponding to a coefficient of friction (COF) of 0.28 (Figure 2a). Upon adsorption of a SCF, friction forces appeared almost two times lower than on Au-coated crystals with a COF of 0.19, and linearity broke down at normal forces above 14 nN. Note that the negative friction forces at a normal force of 1.5 nN represent the known limitation of AFM to measure very low friction forces.^[19] However, measurements on S-SCFs with recombinant SUPs clearly showed lower friction forces (Figure 2b). Linearity corresponding to a COF of 0.08 persisted up to a normal force of 20 nN for K36, while linearity ($R^2 = 0.94$) corresponding to an extremely low COF of 0.06 existed over the entire range of normal forces applied for K72-modified films, indicative of a high structural integrity. Contact of the AFM colloidal probe with the Au-coated quartz crystal (Figure 2c) shows a hard material compared with the softer S-SCFs due to long-range repulsive forces between S-SCFs and the approaching colloidal probe. The repulsive force range arising from the S-SCFs increased with the number of positive charges after adsorption of recombinant cationic SUPs (Figure 2c). To gain more insight into the structural composition of the S-SCFs, XPS was applied to measure the degree of glycosylation, which is related to the water content of the surface (Figure 2d and Supporting

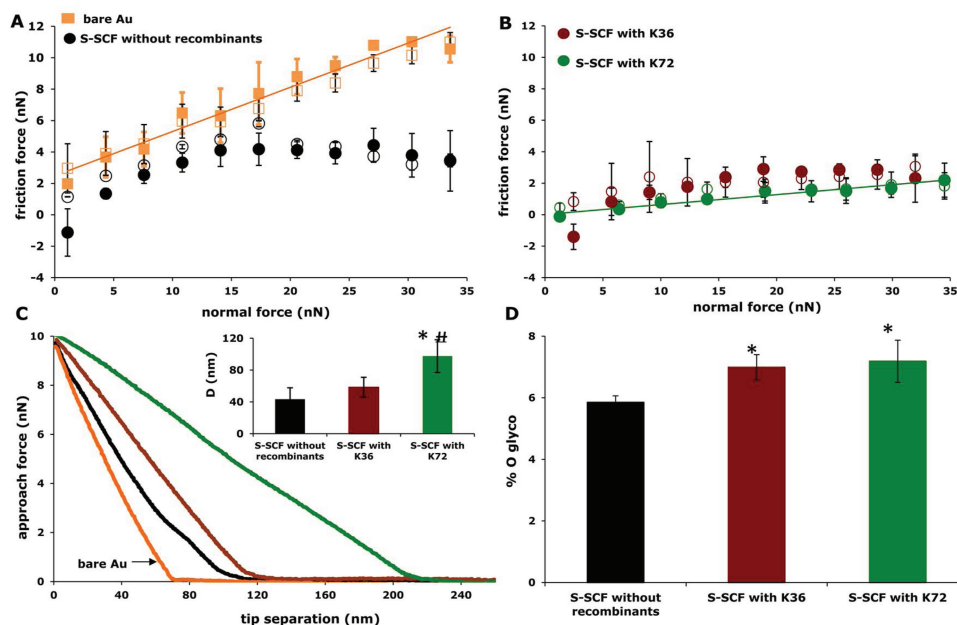


Figure 2. Influence of adsorption of recombinant cationic SUPs and renewed exposure to saliva on the friction forces, repulsive force upon approach and glycosylation of salivary conditioning films. (a, b) Friction force as a function of normal force during increasing (closed symbols) and decreasing (open symbols) normal forces: a) bare Au-coated QCM crystal and S-SCF without adsorbed recombinant cationic SUPs; b) S-SCFs with adsorbed recombinant cationic SUPs K36 or K72 and after renewed exposure to salivary proteins. Error bars represent standard deviations over 12 measurements. c) Example of the repulsive force as a function of tip separation distance for bare Au-coated QCM crystals, S-SCF without adsorbed recombinant cationic SUPs and with adsorbed K36 or K72. The repulsive force range (D) for all adsorbed protein films is calculated with respect to hard contact recorded on bare Au-coated crystal surface (inset Figure 2c). Error bars represent standard deviations over 30 force curves. d) The degree of glycosylation (%O_{glyco}) for S-SCFs without adsorbed recombinant cationic SUPs and with adsorbed K36 or K72, obtained from a decomposition of the O_{1s} photoelectron peak in XPS. Error bars represent the standard deviations over three independent XPS measurements on separately prepared samples. Statistically significant ($p < 0.05$, two tailed Student t-test) differences in repulsive force range (c) and glycosylation (d) of S-SCF with K36 or K72 with respect to S-SCF in absence of adsorbed recombinant SUPs are indicated by *. Differences in repulsive force range between S-SCF with adsorbed K36 or K72 are indicated by #.

Information Table S1). Glycosylation in the S-SCFs with no adsorbed SUPs amounts to $5.8 \pm 0.8\%$ and increases with the molecular weight of the adsorbed SUPs to $6.9 \pm 0.3\%$ and $7.2 \pm 0.6\%$ in SCFs with K36 and K72, respectively.

From the measurements described above one can conclude that cationic recombinant SUPs adsorb on SCFs and decrease their structural softness, i.e. increase their rigidity. SUPs carrying more positive charges create more rigid films, and more efficiently recruit salivary proteins to form a SCF with a thicker globular structure and higher degree of glycosylation, generating a longer repulsive force range and more stable, low friction. Patients with oral dryness symptoms have reduced salivary flow rates, but naturally occurring salivary proteins are always present. This study is the first in which naturally occurring salivary proteins are recruited through the adsorption of recombinant, cationic SUPs to improve several parameters crucial for effective biolubrication. Our approach represents a groundbreaking strategy for artificial biolubrication, where additives act in concert with and enhance the natural lubricants rather than simply replacing them. A proof of principle was obtained for oral lubrication the most challenging environment for biolubrication, but similar recruitment mechanisms may be applied in other parts of the human body as well.

SCF is composed of glycosylated, high-molecular weight mucins (0.25 to 20 MDa) that adsorb in loops and trains (with a

molecular aspect ratio of up to $1/1000$)^[20] and thereby provide a scaffold to hold and retain water molecules at the surface, while adsorbed smaller proteins like proline-rich proteins, histatins, lysozymes, and amylases may be found underneath the loops and between the trains (Figure 3a).^[21] Based on the measurements presented above, we suggest a model for the interaction of cationic SUPs with an existing SCF and for how the adsorbed cationic proteins may be further involved in the recruitment of salivary proteins during renewed exposure to saliva. K36 and K72 bind to the negatively charged mucins leading to elimination of electrostatic stabilization of the adsorbed film and its subsequent collapse, forming a rigid structure (Figure 3b). K72 is a polypeptide consisting of more than 400 amino acids with 72 positively charged groups evenly distributed along the polymer backbone. The higher number of positive charges in K72 compared with K36 neutralizes negative charges in the SCF, but importantly not all positive charges of K72 are engaged in interaction with negative charges in the SCF and positive charges of adsorbed K72 remain available for further interaction with negative charges. This can be concluded from zeta potential measurements (Figure S5), showing more positive charge on SCFs exposed to K72 than on K36-treated and untreated SCFs. Thus uncompensated positive surface charges of adsorbed K72 on a SCF can trigger further recruitment of negatively charged glycosylated mucins during renewed exposure to saliva (Figure 3b;

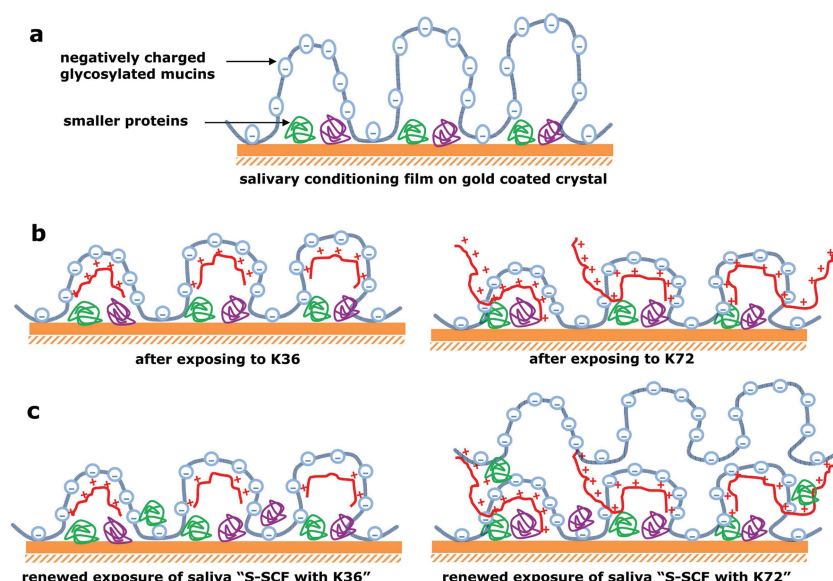


Figure 3. Architecture of SCFs after adsorption of recombinant cationic SUPs with different numbers of positive charges and renewed exposure to saliva. a) Adsorbed salivary conditioning film, showing glycosylated mucins adsorbed in loops and trains over a layer of adsorbed densely packed low-molecular weight proteins, including proline-rich proteins, histatins and lysozymes. b) Salivary conditioning films after adsorption of K36 (left panel) and K72 (right panel). Recombinant cationic SUPs interact with the negatively charged glycosylated mucins, causing collapse of the glycosylated structure through electrostatic interaction. In case of K72, not all positive charges engaged with the mucins and remain available for further interaction (right panel). c) Salivary conditioning films with adsorbed cationic SUPs and after renewed exposure to saliva. No mucins are recruited in the presence of adsorbed K36 (left panel), but remaining positive charges in the film possessing adsorbed K72 recruit mainly glycosylated mucins to form a soft mucinous layer over a compact SCF (right panel).

right panel), resulting in a softer highly hydrated over-layer (Figure 3c). This recruitment process rejuvenates the film, as it can bind to more water molecules. This is one critical step beyond simply restoring the film structure, as observed in SCFs formed after reflow of saliva over K36-treated and buffer-treated SCFs. Note that an analogous layer-by-layer assembly of bovine mucins using chitosan as a cationic polyelectrolyte has been demonstrated previously.^[22]

The rigid and hydrated S-SCF, modified with adsorbed K72 and after renewed exposure to saliva, shows low friction forces and a structural integrity that is not compromised at higher contact pressures, in contrast to films containing K36 or untreated films. The breakdown of structural integrity in these latter films can be seen from the discontinuity in the linearity of friction force against the normal force.^[23,24] In order to determine the mechanical strength of the S-SCFs, we have applied Von Mises distortion energy criterion that relates the normal force at which the discontinuity arises (f_L) to the yield strength (σ_y) of the films^[24,25] through

$$\sigma_y = \frac{1}{\sqrt{2}} \left[2 \left(\frac{3 * f_L}{2 * \pi * R_{tip} * \delta} \right)^2 + 6 \left(\frac{3 * f_f}{2 * \pi * R_{tip} * \delta} \right)^2 \right]^{0.5} \quad (1)$$

where R_{tip} is the radius of the colloidal probe (2.37 μm), δ is the elastic displacement of the film determined from a Hertzian fit to the force-distance curves as obtained by colloidal probe

AFM and f_f is the friction force at f_L . Accordingly, yield strength for S-SCF in absence of recombinant cationic SUPs is 80 ± 12 kPa, increasing to 102 ± 8 kPa in the presence of adsorbed K36. In contrast, no discontinuity in the linearity of friction force against the normal force was observed for K72-treated films within the range of normal forces applied, indicating that the yield strength of S-SCFs in presence of K72 exceeds 102 kPa. This increase in the yield strength can be attributed to improved cohesive strength in the adsorbed films, against the applied shear force.

In conclusion, an ideal biolubricant-like artificial saliva should lubricate the oral surfaces and at the same time sustain this lubrication for lasting benefits. Here we demonstrate that non-toxic, recombinant cationic SUPs adsorb on SCFs to recruit further glycosylated mucins from saliva, provided the number of positive charges is sufficiently high. These hydrated and rigid films improve interfacial lubrication and maintain their structural integrity upon high contact pressures. Current generations of artificial salivas are inadequate to restore oral lubrication on a lasting basis. Cationic recombinant SUPs as additives, however, go even beyond restoration to rejuvenation of the film, affording effective lubrication under conditions of reduced availability of naturally occurring

proteins. On the basis of the cooperative layer-by-layer mechanism laid out here, cationic protein polyelectrolytes show great promise for restoring impaired biolubrication.

Experimental Section

Design, development, and characterization of recombinant cationic polypeptides: All chemicals were used as received without any further purification. The pUC19 cloning vector, restriction enzymes, and GeneJET Plasmid Miniprep kit were purchased from Fermentas (St. Leon-Rot, Germany). Digested DNA fragments were purified using QIAquick spin miniprep kits from QIAGEN, Inc. (Valencia, CA). *E. coli* XL1-Blue competent cells for plasmid amplification were purchased from Stratagene (La Jolla, CA). Oligonucleotides for sequencing were ordered from Sigma-Aldrich (St. Louis, MO). A-cyano-4-hydroxycinnamic acid and internal standards trypsinogen and enolase for mass spectrometry were purchased from LaserBio Labs (Sophia-Antipolis, France). Ultrapure water, resistivity >18.2 M Ω -cm was used for all experiments.

Gene oligomerization: Integrity of DNA sequence was verified by sequencing of coding and complementary DNA strand after each cloning step (SequenceXS, Leiden, The Netherlands). SUP monomer gene SUP K9, encoding for the polypeptide [GVGVP(GKGVPS)]₉, was ordered from Entelechon (Regensburg, Germany) and was delivered in the pEN vector. As the recognition sites of restriction enzymes PflMI and BglII had to be preserved, one valine residue per ten pentapeptide repeats was incorporated instead of a lysine residue during each oligomerization step. All cloning steps were performed according to standard molecular biology methods. SUP K9 was transferred into the standard cloning vector pUC19, digested with EcoRI and HindIII. Gene

oligomerization was performed as described by Meyer and Chilkoti.^[26] Genes of correct length were identified by gel electrophoresis following plasmid digestion with EcoRI and HindIII and sequencing (ServiceXS, Leiden, The Netherlands).

Protein expression and purification: Genes coding for K36 and K72 were cloned into the expression vector pET25b(+)-SfiIHis6 as described before.^[18] *Escherichia coli* BLR (DE3) (Novagen Inc., San Diego, CA) were transformed with pET25b(+)-SfiIHis6 containing the respective SUP genes. For protein production, Terrific Broth medium (TB; 12 g/L tryptone, 24 g/L yeast extract) enriched with phosphate buffer (2.31 g/L KH_2PO_4 , 12.54 g/L K_2HPO_4) and glycerol (4 mL/L), and supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin was inoculated with an overnight starter culture to an initial optical density at 600 nm (OD_{600}) of 0.1 and incubated at 37 °C with orbital agitation at 250 rpm until OD_{600} reached 0.7. Cultures were shifted to 30 °C, for additional 16 h. Cells were harvested by centrifugation (7000 g, 20 min, 4 °C), resuspended in lysis buffer (10 mM TrisHCl, pH 8.0, 300 mM NaCl, 20 mM imidazole) to an OD_{600} of 100 and disrupted with a constant cell disrupter (Constant Systems Ltd., Northants, UK). Cell debris was removed by centrifugation (40000 g, 90 min, 4 °C). Polypeptides were purified from the supernatant under native conditions by Ni-sepharose chromatography (GE Healthcare). Protein-containing fractions were dialyzed extensively against ultrapure water. Purified polypeptides were frozen in liquid nitrogen, lyophilized and stored at -17 °C until further use.

Protein characterization: Concentrations of purified SUPs were determined by measuring absorbance at 280 nm on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Protein purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel according to Laemmli.^[27] Gels were stained with coomassie staining solution (40% methanol, 10% glacial acetic acid, 1 g/L Brilliant Blue R250). Photographs of the gels were taken with a LAS-3000 Image Reader (Fuji Photo Film GmbH, Dusseldorf, Germany). Both K36 and K72 showed reduced electrophoretic mobility compared to a commercial molecular weight standard, a well-known phenomenon for elastin-like polypeptides.^[26,28] Mass spectrometric analysis was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) in the linear positive mode. The protein samples were mixed 1:1 v/v with a recrystallized α -cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% acetonitrile and 0.1% trifluoro acetic acid, LaserBio Labs). Mass spectra were analyzed and calibrated internally with the Data Explorer software, version 4.9 (Applied Biosystems, Foster City, CA, USA). Trypsinogen (MW = 23,980) and enolase (MW = 46,672) were used as calibration standards for K36 (expected MW = 18,888) and K72 (expected MW = 36,313), respectively.

Saliva collection: Saliva from twenty healthy volunteers (10 men, 10 women, average age 30 ± 8 years) was collected into ice-chilled cups after stimulation of flow by chewing Parafilm®. Volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical-Ethical-Committee at the University-Medical-Center-Groningen, The Netherlands. After saliva was pooled and centrifuged at 12000 g, 15 min, 4 °C, phenylmethylsulfonylfluoride was added to a concentration of 1 mM as a protease-inhibitor. The solution was again centrifuged, dialyzed for 24 h, 4 °C against demineralized water, and freeze-dried for storage. Lyophilized stock was prepared by mixing freeze-dried material originating from 2 L of saliva. Reconstituted saliva was prepared from the lyophilized stock by dissolution of 1.5 mg/mL in buffer (2 mM potassium phosphate, 1 mM CaCl_2 , 50 mM KCl, pH 6.8).

Quartz crystal microbalance with dissipation monitoring: Structural softness and formation kinetics of SCFs were studied using a QCM-D device, model Q-sense E4 (Q-sense, Gothenburg, Sweden). Au-coated quartz crystals with 5 MHz were used as substrata. Before each experiment, crystals were cleaned by 10 min UV/ozone treatment, followed by immersion into a 3:1:1 mixture of ultrapure-water, NH_3 and H_2O_2 at 70 °C for 10 min, drying with N_2 and another UV/ozone treatment. QCM-D chamber is disc-shaped with the inlet and outlet facing the crystal surface. The chamber was perfused with buffer by peristaltic pump (Ismatec SA, Glatbrugg, Switzerland), when stable

base lines for both frequency and dissipation at third harmonics were achieved, saliva was introduced. Saliva was perfused through the chamber at 25 °C for 2 h, flow rate of 50 $\mu\text{L}/\text{min}$, corresponding with a shear rate of 3 s^{-1} after which, the chamber was perfused with buffer or 0.05% w/v of SUP for 2 min and followed by another 2 h of salivary flow to form a secondary SCF denoted as S-SCF. In between steps, the chamber was perfused with buffer for 15 min or till a stable frequency shift of less than 2 Hz over 10 min was observed. The shear rate in the QCM-D represents a low oral salivary flow.^[29] Frequency and dissipation were measured real-time during perfusion. After experiments, crystals were removed from the QCM-D and immediately used for further experiments.

Colloidal probe atomic force microscopy: Friction force, surface topography and repulsive force range toward a colloidal AFM probe^[30] were measured in buffer with an AFM (Nanoscope IV Dimension™ 3100) equipped with a Dimension Hybrid XYZ SPM scanner head (Veeco, New York, USA) on the differently adsorbed SCFs. Rectangular, tipless cantilevers were calibrated for their torsional and normal stiffness using AFM Tune IT v2.5 software.^[31] The normal stiffness (K_n) was between 0.01–0.04 N/m and the torsional stiffness (K_t) between $2-4 \times 10^{-9}$ Nm/rad. Subsequently, a silica-particle of 4.74 μm diameter (d) (Bangs laboratories, Fishers, IN, USA) was glued to a cantilever with an epoxy glue (Pattex, Brussels, Belgium). The deflection sensitivity (α) of the colloidal probe was recorded at a constant compliance with bare crystal in buffer to calculate the normal force (F_n) applied using

$$F_n = \Delta V_n * \alpha * K_n \quad (2)$$

where ΔV_n is the voltage output from the AFM photodiode due to normal deflection of the colloidal probe. The torsional stiffness and geometrical parameters of the probe were used to calculate the friction force (F_f)^[19,32] according to

$$F_f = \frac{\Delta V_L * K_t}{2 * \delta * (d + t/2)} \quad (3)$$

where t is the thickness of the cantilever, δ is the torsional detector sensitivity of the AFM and ΔV_L corresponds to the voltage output from the AFM photodiode due to lateral deflection of the probe. Lateral deflection was observed at a scanning angle of 90 degrees over a scan area of $5 \times 5 \mu\text{m}^2$ and a scanning frequency of 1 Hz. The colloidal probe was incrementally loaded and unloaded up to a normal force of 35 nN. At each normal force, 10 friction loops were recorded to yield the average friction force. Repulsive force-distance curves between a colloidal probe and the films were obtained at a trigger threshold of 10 nN and an approach and retraction velocity of 10 $\mu\text{m}/\text{s}$. The repulsive force range (D) was determined at a point where colloidal tip starts experiencing the repulsive force >1 nN between the two interacting surfaces.

X-ray photoelectron spectroscopy: Glycosylation of the adsorbed SCFs was determined by using XPS (S-probe, Surface Science Instruments, Mountain View, CA, USA). Films adsorbed on Au-coated quartz crystals as removed from the QCM-D, were dried in the pre-vacuum chamber of the XPS, and then subjected to a vacuum of 10^{-7} Pa. X-rays (10 kV, 22 mA, spot size $250 \times 1000 \mu\text{m}$), were produced using an aluminum anode. Scans in the binding energy range of 1–1100 eV were made at low resolution (pass energy 150 eV). The area under each peak was used to yield elemental surface concentrations for C, O, N, and Au after correction with sensitivity factors provided by the manufacturer. The O_{1s} peak was split into three components for oxygen involved in amide groups (C=O-N; 531.3 eV), carboxyl groups (C-O-H; 532.7 eV) and oxygen arising from the crystal. Accordingly, the fraction of the O_{1s} peak at 532.7 eV ($\% \text{O}_{532.7}$) was used to calculate the amount of oxygen involved in glycosylated moieties ($\% \text{O}_{\text{glyco}}$) and amides ($\% \text{O}_{\text{amides}}$).

$$\% \text{O}_{\text{glyco}} = \% \text{O}_{532.7} * \% \text{O}_{\text{total}} \quad (4)$$

$$\% \text{O}_{\text{amide}} = \% \text{O}_{531.3} * \% \text{O}_{\text{total}} \quad (5)$$

where $\% \text{O}_{\text{total}}$ is the total percentage of oxygen.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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